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A cocaine-sensitive *Drosophila* serotonin transporter: Cloning, expression, and electrophysiological characterization

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ABSTRACT **A cocaine-sensitive, high-affinity *Drosophila* serotonin (5-hydroxytryptamine; 5HT) transporter cDNA, denoted dSERT1, was isolated and characterized in oocytes. dSERT1 shows little transport of other monoamines and is Na^+ and Cl^- dependent. Sequence analysis indicates 12 putative transmembrane domains and strong homologies (≈50%) among dSERT1 and mammalian 5HT, norepinephrine, and dopamine transporters. Interestingly, the pharmacological properties of dSERT1, including sensitivity to antidepressants, are more similar to those of mammalian catecholamine transporters than to mammalian 5HT transporters. Two-electrode voltage-clamp analysis demonstrated 5HT-induced, voltage-dependent currents. Cloning and characterization of dSERT1 adds significantly to our knowledge of the diversity of 5HT transporters with regard to primary sequence, pharmacological profile, and permeation properties.**

One mechanism for the regulation of synaptic activity is through removal of neurotransmitter from the synaptic cleft via high-affinity transporters located on neuronal and glial plasma membranes. By slowing the removal of neurotransmitter from the cleft, inhibition of these transporters can effectively increase the duration of neurotransmitter action on postsynaptic receptors. Recent cloning studies indicate that neurotransmitter transporters consist of two distinct multigene superfamilies. The members of one family are dependent on sodium and chloride for full activity (Na^+/Cl^- transporters) and their substrates include γ-aminobutyric acid (GABA) and glycine (1–7), the monoamine neurotransmitters (8–14), proline (15), taurine (16), betaine (17), and creatine (18). A second superfamily consists of transport proteins that require sodium and potassium for full activity (Na^+/K^+ transporters) and includes the transporters for glutamate (19–21) and certain neutral amino acids (22, 23). Although there is no discernible sequence homology between these two superfamilies, they do share several important features: (*i*) they transport their substrates with high affinity; (*ii*) their activity is dependent on membrane potential; and (*iii*) they are predicted to be integral membrane proteins with multiple transmembrane domains ranging from 6 to 10 for the Na^+/K^+ transporters to 12 for the Na^+/Cl^- transporters.

Although the neurotransmitter transporters cloned to date have all been mammalian, histochemical experiments suggest that members of both of these gene superfamilies are present in a variety of phyla, including crustaceans (24), nematodes (25), and insects (26). The fruitfly *Drosophila* is a particularly useful model for studying neurotransmitter molecular biology, since its nervous system is well-characterized at the anatomical, neurochemical, and genetic levels. *Drosophila* use GABA, acetylcholine, glutamate, dopamine, and serotonin (5-hydroxytryptamine; 5HT) as neurotransmitters and also may use such diverse substances as histamine, octopamine, and tyramine (reviewed in ref. 27). It is likely that transporters for all of these substances exist in the *Drosophila* nervous system. In fact, *in vivo* uptake mechanisms have been suggested to be responsible for 5HT accumulation in *Drosophila* mutants lacking the gene encoding dopa decarboxylase (*Ddc*) (28).

We have used a PCR-based cloning strategy to isolate a *Drosophila* 5HT transporter (termed dSERT1) that is homologous to the mammalian monoamine transporters, as well as to other mammalian members of the Na^+/Cl^- superfamily. We show that dSERT1 possesses a number of functional properties of this family, including a dependence on sodium and chloride, a high affinity for 5HT, and electrogenicity. We also observe that dSERT1 is cocaine sensitive, possessing an unusual pharmacological profile with properties more similar to mammalian catecholamine transporters than to indoleamine transporters, and has electrogenic properties similar to the mammalian GABA transporter.‡

EXPERIMENTAL PROCEDURES

PCR. Degenerate oligonucleotide primers corresponding to two highly conserved amino acid regions near the first (NVWRFPY) and sixth (DAATQIF) transmembrane domains of the rat GABA transporter (1) were designed using *Drosophila* codon usage. The primers consisted of a 144-fold degenerate upstream (sense) primer corresponding to bases 345–363 of GAT1 with the sequence (5′ to 3′) GAG CTC GTC GAC AA(TC) GT(GCT) TGG (CA)G(GCTA) TTC CC(CAG) TA and a 48-fold degenerate downstream (antisense) primer corresponding to bases 1008–1026 of GAT1 with the sequence GGG CCC TCT AGA AA(GA) ATC TG(GC) GT-(GAC) GC(GA) GC(AG) TC. (The underlined portions of these sequences indicate the addition of 5′ restriction sites for cloning purposes.) With these primers, the PCR will generate a 683-bp product from GAT1 mRNA and a 699-bp product from NET1 mRNA. The amplification protocol consisted of 1 min at 94°C, 2 min at 65°C, and 2 min at 72°C for 25–30 cycles. Gel electrophoresis indicated a band of ≈700 bp, which was subcloned into pBluescript SK(−).

Isolation and Characterization of dSERT1 cDNA Clone. The 700-bp product was used in a PCR to synthesize a ^{32}P-labeled probe. This probe was used to screen 1×10^6 phage recombinants from the *Drosophila* head cDNA library in λEXLX (29). After overnight hybridization at 42°C [6× SSPE (1× SSPE = 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA)/35% formamide], the filters were washed at a final stringency of 50°C in 2× standard saline citrate (SSC)/0.1% SDS. Positive clones were rescued and the insert of one of the three positive clones was subcloned in pGEM-7Zf(+) (Promega) for sequencing and *in vitro* transcription.

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Abbreviations: GABA, γ-aminobutyric acid; 5HT, serotonin.

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‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. U02296).

***Xenopus* Oocyte Expression and Transport Assays.** dSERT1 linearized with *Cla* I was used as template for an *in vitro* transcription reaction (Ambion MegaScript kit, Ambion, Austin, TX). To obtain expression levels large enough to measure the transporter-associated currents, we used a previously described PCR-based procedure (30). Methods for oocyte preparation, injection, flux assays, and electrophysiology are described elsewhere (31).

Northern Blot Analysis. Twenty micrograms of total RNA from *Drosophila* head, body, and embryo was separated on a 1% formaldehyde agarose gel and transferred to a nylon membrane (GeneScreen*Plus*, DuPont/NEN) and probed with a 506-bp fragment of dSERT1 (48 bp of the C terminus and 468 bp of 3′ untranslated region). The membrane was hybridized overnight at 42°C (50% formamide/10% dextran sulfate/1 M NaCl/1% SDS), washed at 65°C for 15 min (0.1× SSC/1% SDS), and exposed to film for 24 h at −70°C.

RESULTS

Identification of dSERT1 as a 5HT Transporter. Degenerate oligonucleotides corresponding to conserved portions of putative transmembrane regions 1 and 7 of the Na^+/Cl^- transporter superfamily were used to amplify aliquots of a *Drosophila* head cDNA library. An expected product of ≈700 bp was observed, and DNA sequencing confirmed that this PCR product encoded a member of the Na^+/Cl^- superfamily. The insert was used to isolate a full-length cDNA from the same library. Three clones were plaque purified under high-stringency hybridization conditions and one (clone 39a) was chosen for sequencing and expression characterization. mRNA synthesized from this clone was expressed in *Xenopus* oocytes and screened for potential substrates (Fig. 1). Oocytes injected with clone 39a mRNA transported [^{3}H]5HT at 5-fold higher levels than uninjected oocytes when incubated for 30 min in 760 nM [^{3}H]5HT. Uptake of [^{3}H]GABA, [^{3}H]glutamate, [^{3}H]histamine, [^{3}H]dopamine, and [^{3}H]norepinephrine was not significantly over the background levels. This indicates that clone 39a encodes a 5HT transporter, which we have designated dSERT1.

Sequencing and Molecular Characterization of dSERT1. Sequencing of dSERT1 revealed a 2461-bp insert with an open reading frame of 1743 bases. The proposed initiator ATG, at base 109, occurs within a reasonable match to the consensus translation initiation site (32). The open reading frame encodes a protein of 581 amino acids with a predicted mass of 67 kDa. This sequence does not contain an in-frame stop codon or an alternative initiation codon in the 108-bp 5′ untranslated region of dSERT1. It is possible that there is an upstream initiation codon in the complete mRNA and that the present cDNA encodes a functional truncated protein. Hydropathy analysis (33) predicts 12 putative membrane-spanning domains, indicating a membrane topology similar to that proposed for all members of the Na^+/Cl^- transporter superfamily (1). There is one potential N-linked glycosylation site, located in the large extracellular loop between the third and fourth transmembrane regions. The primary amino acid sequence predicts four potential protein kinase C phosphorylation consensus sites: two in putative extracellular regions and two in the intracellular N terminus (Thr-3 and Thr-4). Comparison of the predicted amino acid sequence to those of the mammalian 5HT transporters and to the human norepinephrine transporter reveals considerable homology (Fig. 2): 52% and 53% amino acid identity to the rat and human 5HT transporters, respectively (13, 34); 51% identity to NET1 (8); and 48% identity to the rat/bovine dopamine transporters (9–12). The percentage identity is only 39–43% between dSERT1 and the transporters for GABA (1), glycine (5), proline (15), and taurine (16). Comparison of the dSERT1 sequence to sequences in the Genbank data base using the BLAST program revealed no homologous sequences other than members of the Na^+/Cl^- transporter family.

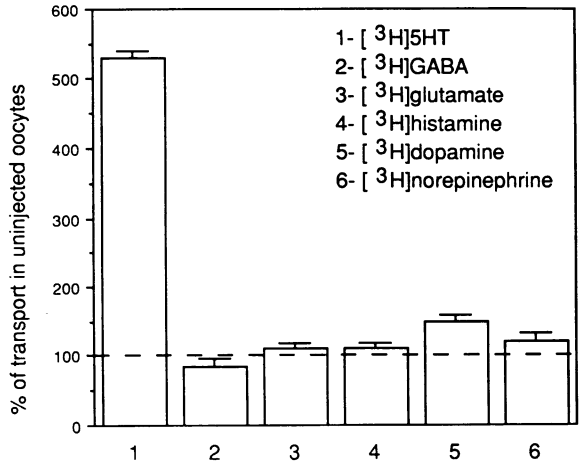

FIG. 1. Transport of various neurotransmitters by dSERT1 expressed in *Xenopus* oocytes. Final concentrations of the listed neurotransmitters were as follows: 760 nM [^{3}H]5HT, 260 nM [^{3}H]GABA, 370 nM [^{3}H]glutamate, 3.8 μM [^{3}H]histamine, 800 nM [^{3}H]dopamine, 1.3 μM [^{3}H]norepinephrine. Each point in these experiments is the mean ± SEM of at least four oocytes.

Northern Blot Analysis. A Northern blot revealed a strong signal at ≈3.3 kb in total RNA from adult *Drosophila* heads, but no detectable signal with RNA from adult *Drosophila* bodies or embryos with an 18-h exposure (data not shown). Thus, dSERT1 is predominantly expressed in structures associated with the head, most likely neurons and/or glia of the cephalic nervous system (35).

Transport Studies. Oocytes injected with dSERT1 mRNA exhibit an ion-dependent, high-affinity 5HT uptake system that saturates with increasing 5HT concentration (Fig. 3). Eadie–Hofstee analysis (Fig. 3 *Inset*) demonstrates a K_m of 637 ± 100 nM (mean ± SEM of four experiments), which is similar to the K_m values determined for the cloned and endogenous mammalian 5HT transporters (13, 14, 34, 36–39). High-affinity transport of 5HT by dSERT1 is dependent on extracellular Na^+ and Cl^-, a hallmark of this transporter family (40). Substitution of Na^+ with either Li^+ or choline reduced transport by ≈95%, while substitution of Cl^- with nitrate or acetate inhibited uptake by 85–91% (data not shown).

Pharmacological Specificity. Drug sensitivity of dSERT1 was assessed by compounds known to inhibit mammalian biogenic amine transport (Table 1). The antidepressants fluoxetine and clomipramine were 6- to 71-fold lower in potency at dSERT1 than at the mammalian 5HT transporters. By contrast, mazindol and nomifensine, which are strong inhibitors of NET1 and DAT1, but are less effective at the mammalian 5HT transporter, were potent inhibitors of dSERT1. Thus, with respect to this group of antidepressants, dSERT1 has a pharmacological profile more similar to the mammalian catecholamine transporters than to the mammalian 5HT transporters. This characteristic is further underscored by the observation that dSERT1 is ≈5-fold more sensitive to cocaine, a potent inhibitor of mammalian dopamine and norepinephrine uptake, than are the mammalian 5HT transporters. However, the sensitivity of dSERT1 to amphetamine is closer to that observed for the rat and human 5HT transporters than to the human norepinephrine transporter, possibly because amphetamine is a substrate for 5HT transporters. Therefore, the pharmacological profile of dSERT1 represents a mixture of those seen for the mammalian biogenic amine transporters. It is important to note, however, that the specificity of dSERT1 for 5HT as a

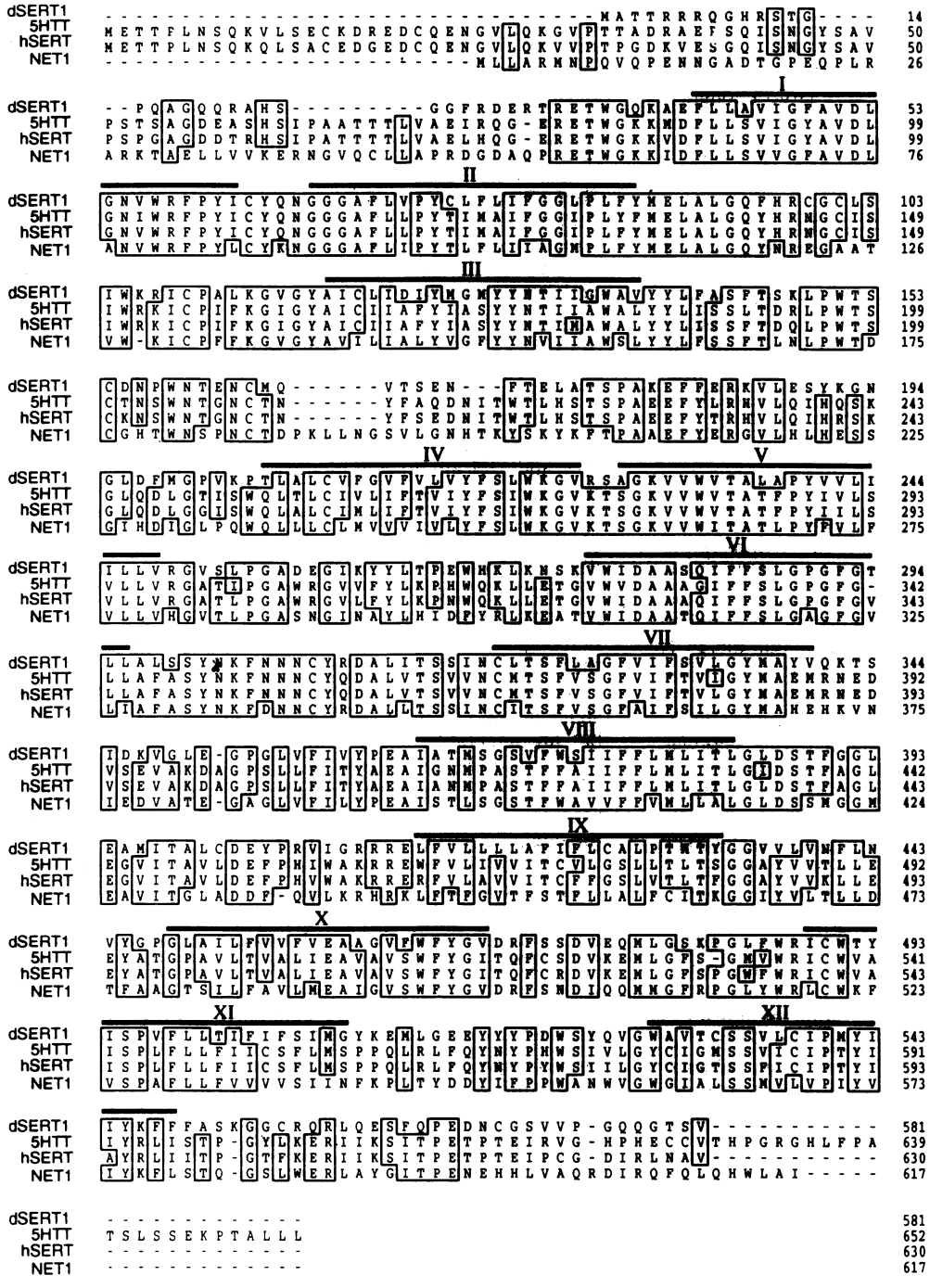

FIG. 2. Alignment of amino acid sequences of dSERT1, rat 5HT transporter (5HTT), human 5HT transporter (hSERT), and human norepinephrine transporter (NET1). Alignments were based on a Dayhoff matrix using the PILEUP program of the Genetics Computer Group sequence analysis software package (Madison, WI). Boxes designate identical or conservatively substituted amino acids in all transporter sequences. Putative transmembrane domains for dSERT1 are denoted by horizontal bars with Roman numerals.

substrate is quite high; unlabeled 5HT is 158- to 479-fold more potent than epinephrine, dopamine, and norepinephrine in reducing [^{3}H]5HT uptake by dSERT1-injected oocytes. This finding corroborates the results of uptake experiments with labeled catecholamines (Fig. 1) and further establishes that dSERT1 is indeed a 5HT transporter rather than a substrate-ambiguous catecholamine transporter.

We searched for additional organic substrates of dSERT1 by examining their ability to decrease transport of 100 nM [^{3}H]5HT. Tryptamine, which is a substrate for the endogenous platelet 5HT transporter (41), was the most effective inhibitor tested in this series, reducing accumulation of [^{3}H]5HT to 27% ± 5% of control values (mean ± SEM) at 200 μM. Tryptophan, a precursor of tryptamine and 5HT, did not decrease 5HT transport (105% ± 5.4%) at 200 μM, nor did the metabolite of 5HT, 5-hydroxyindoleacetic acid (100% ± 13%) at 100 μM. Tyramine, a putative insect neurotransmitter, reduced uptake to 61% ± 7% of control values at 200 μM. Octopamine and histamine, two other putative insect neurotransmitters, had little or no effect on [^{3}H]5HT transport at 200 μM (82% ± 15% and 95% ± 15%, respectively). For comparison, nonradioactive 5HT (acting as a diluent) reduced transport of [^{3}H]5HT by ≈50% at 100 nM, as expected.

Ion and Voltage Dependence of Transport-Associated Currents. By measurements in individual voltage-clamped oocytes, we found that uptake of [^{3}H]5HT is voltage dependent; for example, in one set of measurements over a 5-min time period, uptake increased from 34 ± 16 fmol per oocyte to 90 ± 27 fmol per oocyte between −40 and −80 mV (data not shown). This result prompted us to ask whether dSERT1 is an electrogenic transporter. We used two-electrode voltage-clamp analysis to characterize 5HT-induced currents. When oocytes expressing dSERT1 were exposed to 5HT concentrations of 0.1–30 μM, distinct inward currents could

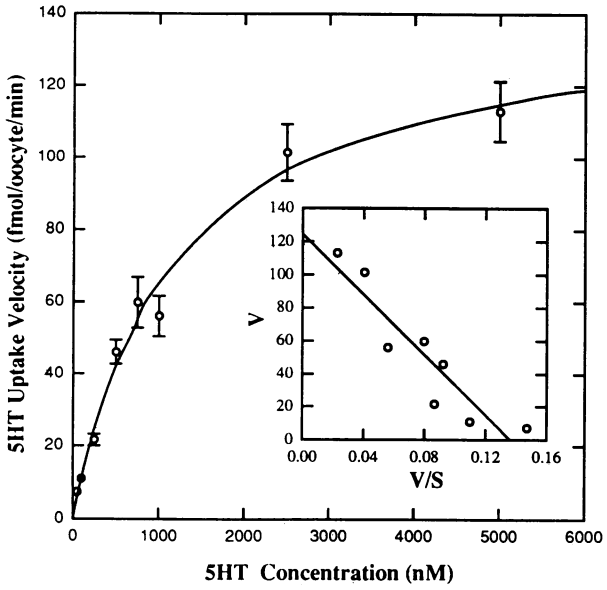

FIG. 3. Kinetics of [^{3}H]5HT uptake. Data for these experiments are representative of four separate experiments and each point is the mean ± SEM of seven determinations. (*Inset*) Eadie–Hofstee plot of initial velocity data.

be measured (Fig. 4*A*). At saturating concentrations of 5HT and membrane holding potentials of −60 or −80 mV, the largest currents induced were ≈300 nA. The magnitude of the 5HT-induced inward currents remained essentially constant during 5HT application times as long as 5 min (n = 11). The 5HT-induced currents were observed in dSERT1-expressing oocytes from three separate oocyte batches but were not seen in uninjected oocytes (n = 9) or in control oocytes injected with mRNA encoding the rat brain GABA transporter GAT1 (n = 6). Application of 10 μM dopamine, norepinephrine, and epinephrine did not evoke measurable currents (data not shown), further indicating the specificity of dSERT1 for 5HT.

Fig. 4*B* shows the current–voltage relationship for the 5HT-induced current and the effect of ion substitution on these currents. For an oocyte superfused with 5 μM 5HT in ND96 medium, the 5HT-induced current increased more than linearly between +40 and −100 mV. Between −40 and −80 mV, there was a 2.4-fold increase in the amount of transport-associated current, similar to the voltage dependence of [^{3}H]5HT uptake. When either Na^+ or Cl^- is eliminated from the superfusion medium, the 5HT-induced currents are too small for systematic current–voltage analysis.

DISCUSSION

When expressed in *Xenopus* oocytes, dSERT1 reconstitutes a high-affinity, cocaine-sensitive, Na^+- and Cl^--dependent 5HT uptake system. The apparent K_m for 5HT of dSERT1 (637 nM) is comparable to the range of values determined for the cloned mammalian 5HT transporters (13, 14, 34) as well as for native mammalian 5HT transporters (36–39). It has a low affinity for norepinephrine, dopamine, and epinephrine and does not transport other neurotransmitters found in the *Drosophila* nervous system (e.g., glutamate, GABA, dopamine, or histamine). Although the amino acid sequence of dSERT1 shows various degrees of homology with all known members of the Na^+/Cl^- transporter superfamily, it is most closely related to the mammalian 5HT, norepinephrine, and dopamine transporters. The observation that dSERT1 has about the same degree of structural relatedness to the three mammalian monoamine transporters correlates with its unusual functional properties. Its sensitivity to antidepressants more closely resembles that of NET1 and DAT1 than that of the mammalian 5HT transporter. Similarly, dSERT1 is more sensitive to cocaine than is the mammalian 5HT transporter. By contrast, the sensitivity of dSERT1 to amphetamine is more similar to that of the mammalian 5HT transporters than to that of NET1 and DAT1. Thus, although dSERT1 has a high substrate specificity for 5HT, it exhibits a pharmacological profile that shares features of both mammalian indoleamine and catecholamine transporters.

External application of 5HT to dSERT1-injected oocytes induces an inward current that is associated with 5HT uptake. This indicates that the transporter encoded by dSERT1 is electrogenic and that the net charge movement associated with transport is inward. Furthermore, (*i*) the substrate-induced current does not inactivate, even with extended substrate application times; (*ii*) the substrate-induced current has an absolute requirement for external Na^+; (*iii*) the substrate-induced current is strongly dependent on external Cl^-; (*iv*) the current–voltage relationship shows

Table 1. Comparison of IC_{50} values

Inhibitor	5HT transporters, nM			Norepinephrine transporter, nM	Dopamine transporter, nM
	dSERT1 (*Drosophila*)	5HTT* (rat)	hSERT† (human)	NET1‡ (human)	DAT§ (rat)
Mazindol	27	548	NA	1.4	27
Nomifensine	44	NA	100	7.7	118
5HT	165	NA	NA	>10,000	>10,000
Cocaine	184	1,080	1,000	140	336
Fluoxetine	450	33	6	NA	NA
Desipramine	2,217	1,680	250	3.9	NA
Clomipramine	2,670	7.1	NA	NA	NA
Amphetamine	10,800	3,180	20,000	56.1	881
Epinephrine	26,000	NA	NA	NA	>1,000
Dopamine	42,000	>10,000	NA	139	316
Norepinephrine	79,000	NA	NA	NA	>10,000

Final [^{3}H]5HT concentrations were 100 nM, 50–100 nM, and 20 nM in determinations for dSERT1, 5HT transporter (5HTT), and human SERT1, respectively. Final [^{3}H]norepinephrine concentration was 20 nM for NET1 and final [^{3}H]dopamine concentration was 15 nM for dopamine transporter (DAT). NA, not applicable.

*Hoffman *et al.* (13).

†Ramamoorthy *et al.* (34).

‡Pacholczyk *et al.* (8).

§Giros *et al.* (12).

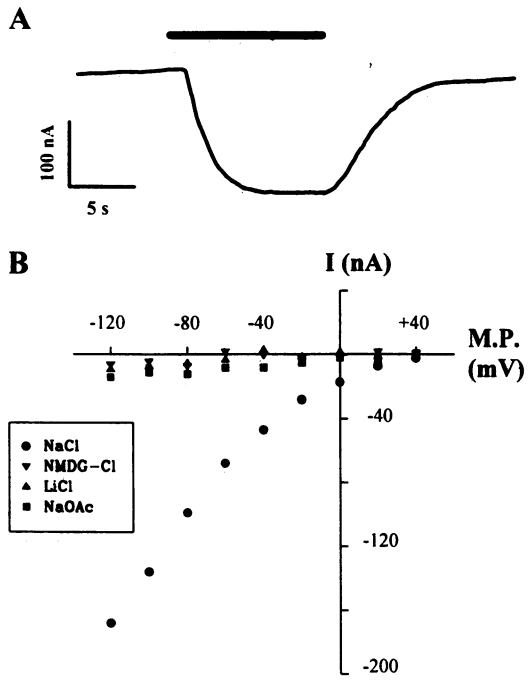

FIG. 4. Ion and voltage dependence of 5HT-induced inward currents in oocytes expressing dSERT1. (*A*) Transport-associated inward current for an oocyte voltage clamped at a holding potential of −60 mV. The current was evoked with 10 μM 5HT superfused in ND96 medium for the time indicated by the bar above the trace. (*B*) Steady-state currents, in the presence and absence of Na^+ and Cl^- in the assay medium, plotted as a function of voltage for a second oocyte. The 5HT concentration was 5 μM.

a greater than linear increase in inward current with membrane potential in the range of 0 to −80 mV. These electrophysiological properties of dSERT1 are similar to those shown for the mammalian GABA transporter GAT1 (42).

It is likely that, as in vertebrates, 5HT plays a range of roles in the fly, both as a classical neurotransmitter and as a neuromodulator (35). Combined with the repertoire of available genetic tools for *Drosophila*, dSERT1 will be an important reagent for further analysis of serotonergic neurons and pathways, for isolation of other insect Na^+/Cl^- transporters, and for analyzing the structural basis of transporter function.

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